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(54) Title: HORMONE RESPONSE ELEMENT COMPOSITIONS AND ASSAY

| | FINGER 1 | | | LINKER | | FINGER 2 | | | | MTV | $TRE_p$ | ERE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | | 5 | 6 | 7 | 8 9 | | | |
| hGRnx | CLVC | SDEASGCHYGVLT | C | GSC KV | FFKRAVEG--QHNYL | C | AGRND | C | IIDKIRRKNCPA CRYRKC LQAGM | 100 | - | - |
| GTG | .V.. | G.K.T.Y..RCI. | . | EG. .G | ..R.TIQKNLHPS.S | . | KYEGK | . | V...VT.NQ.QE ..FK.. IYV.. | - | 100 | 100 |
| GTG8B | .... | ..... | . | EG. .G | .....--..... | . | KYEGK | . | ..... ..... ..... | - | 97 | 330 |
| | | | | P | | | D | | | | | |
| GTG3A | .... | ..... | . | EG. .G | .....--..... | . | ..... | . | ..... ..... ..... | - | - | 250 |
| GTG2 | .... | ..... | . | EG. .. | .....--..... | . | ..... | . | ..... ..... ..... | 18 | - | 320 |
| GTG1 | .... | ..... | . | EG. .. | .....--..... | . | ..... | . | ..... ..... ..... | 110 | - | 40 |

(57) Abstract

The present invention discloses steroid/thyroid hormone receptor DNA binding domain compositions that determine target gene specificity. The invention further discloses methods converting the target gene specificity of one receptor into the target gene specificity of another. Still further the invention discloses novel assays for identifying ligands for orphan hormone receptors. These assays are especially useful since they avoid the necessity of constructing chimeric genes and proteins in order to search for ligands that can activate a putative receptor.

# HORMONE RESPONSE ELEMENT COMPOSITIONS AND ASSAY

## FIELD OF THE INVENTION

The present invention relates generally to hormone receptor genes and proteins. More particularly, the present invention relates to identification and characterization of determinants that govern target gene specificity for the superfamily of steroid/thyroid hormone receptors. In addition the invention relates to novel assays for identifying ligands for "orphan" hormone receptors.

## BACKGROUND OF THE INVENTION

The steroid/thyroid hormone receptors form a superfamily of ligand-dependent transcription factors that influence cell function and fate in eukaryotes. It is known that these receptors transduce extracellular hormonal signals to target genes that contain specific enhancer sequences referred to as hormone-response elements (HREs) (Evans 1988; Green and Chambon 1988). It is also known that each receptor recognizes its own HREs, thus assuring that a distinct response is triggered by different hormones.

Sequence comparisons and mutational analyses of glucocorticoid receptor (GR) have identified functional domains responsible for transcriptional activation and repression, nuclear localization, DNA binding, and hormone binding (Gigure, *et al.*, 1986; Hollenberg, *et al.*, 1987; Rusconi, *et al.*, 1987; Picard and Yamamoto, 1987; Hollenberg and Evans, 1988; Oro, *et al.*, 1988a). The DNA binding domain, which is required to activate transcription, consists of 66-68 amino acids of which about 20 sites, including nine cysteines ($C_1$ to $C_9$), are invariant among different receptors (Fig. 1a). The modular structure of members of this receptor super-

family allows the exchange of one domain for another to create functional chimera. This strategy was used to dem nstrate that the DNA binding domain is solely responsible for the specific recognition of the HRE *in vivo* (Green and Chambon, 1987; Giguere, *et al.*, 1987; Petkovich, *et al.*, 1987; Kumar, *et al.*, 1987; Umesono, *et al.*, 1988; Thompson, *et al.*, 1989) and *in vitro* (Kumar and Chambon, 1988).

By analogy with the proposed structure for *Xenopus* transcription factor TFIIIA (Miller, *et al.*, 1985), the invariant cysteines are thought to form two "zinc fingers" for specific DNA binding. In a polypeptide encompassing the DNA binding domain of rat GR, it has been shown that each of two Zn(II) is coordinated in a tetrahedral arrangement by four cysteines (Freedman, *et al.*, 1988). Involvement of these cysteines in Zn(II) coordination is also supported by the fact that eight out of nine cysteines, enough to chelate two Zn(II), are required for the receptor function revealed by point mutagenesis experiments (Hollenberg, *et al.*, 1988; Severne, *et al.*, 1988).

Functional models have been proposed in an attempt to coordinate research results with gene regulation mechanisms that function *in vivo*. As those skilled in the art will know, one such model for the DNA binding domain is "zinc finger model". (A predicted "finger" structure is presented in Fig. 1b; also *see* Severne, *et al.*, 1988.) In this model, the first four cysteines ($C_1$ to $C_4$) chelate one Zn(II) to form Finger 1, which includes a loop of 13 amino acids between $C_2$ and $C_3$. Finger 2 is formed by the next four cysteines ($C_5$ to $C_8$), since function is retained when the ninth cysteine is changed to an alanine or serine (Severne, *et al.*, 1988). Finger 2 has a loop of 9 amino acids, and is separated by a "Linker" of 15-17 amino acids from Finger 1. Both fingers are functionally required becaus

As the present invention discloses, functional characterization of mutant receptors carrying chimeric DNA binding domains has made it possible to dissect molecular determinants of target gene specificity for GR, ER, and TR. For example, as th present invention discloses, the identity of GR DNA binding domain can be converted into those of ER and TR by changing three and eight amino acids, respectively. The present invention also discloses that a single Gly to Glu change in the first "zinc finger" of the GR receptor produces a receptor with dual HRE (*i.e.*, GRE and ERE) sequence responsiveness. These discoveries localize two structural determinants of target gene specificity and suggest a simple pathway for the co-evolution of receptor DNA binding domains and regulatory networks.

These discoveries also make it possible to convert one receptor into another, and to create engineered receptors that have desired HRE recognition

features. They have also enabled the development of assays that are useful for identifying ligands for "orphan" hormone receptors. Such assays are especially advantageous because they eliminate the necessity of constructing chimeric genes and proteins in order to search for ligands that can activate the orphan receptors.

## BRIEF DESCRIPTION OF THE DRAWINGS

The following is a brief description of the drawings. More detailed descriptions are found in the section of the specification labeled, "Detailed Description of the Drawings".

FIGURE 1 (a-c) is a schematic drawing. Fig. 1a shows a comparison of amino acid sequences among the DNA binding domains of hGR, $hT_3\beta$, and hER. Fig. 1b shows predicted zinc fingers based on those for rat GR18. Fig. 1c shows the structures of optimized hormone response elements for GR (GRE), ER (ERE), and $T_3R$ (TRE).

FIGURE 2 (a and b) is a schematic drawing that illustrates transactivation of luciferase reporter plasmids by mutant receptors.

FIGURE 3 is a schematic drawing that illustrates identification of two distinct elements specifying the TREp+ and ERE+ phenotypes.

FIGURE 4 (a-f) is comprised of the combination of a schematic drawing and photographs of blots. Fig. 4 shows induction of *CAT* activities by mutant receptors from the basal ΔMTV-*CAT* (ΔM), $T_3$-responsive ΔMTV-TREp-*CAT* (TREp), an estrogen-responsive ΔMTV-ERE-*CAT* (ERE) reporters.

## DEFINITIONS

In the present specification and claims, referenc will be made to phrases and terms of art which are expressly defined for use herein as follows:

As used herein, species are identified as follows: h, human; r, rat; m, mouse; c, chicken; and d, *Drosophilia*.

As used herein, "st roid hormone superfamily of receptors" refers to the class of related receptors comprised of glucocorticoid, mineralocorticoid, progesterone, estrogen, estrogen-related, vitamin $D_3$, thyroid, v-*erb-A*, retinoic acid and E75 (*Drosophilia*) receptors. *See* Evans (1988) and the references cited therein.

As used herein, GR means glucocorticoid receptor. The DNA referred to as hGR codes for human glucocorticoid receptor GR. hGR is encoded by plasmid pRShGR which has been deposited for patent purposes and accorded ATCC No. 67200.

As used herein, MR means mineralocorticoid receptor. The DNA referred to as hMR codes for human mineralocorticoid receptor MR. hMR is encoded by plasmid pRShMR which has been deposited for patent purposes and accorded ATCC No. 67201.

As used herein, TR means thyroid receptor and $T_3R$ means triiodothyronine ($T_3$) receptor. TR$\alpha$ and TR$\beta$ refer to the *alpha* and *beta* forms of the thyroid receptor. Plasmid pher*b-A* 8.7 encodes hTR$\alpha$; it has been deposited for patent purposes and accorded ATCC No. 40374. Plasmid peA101 encodes hTR$\beta$; it has been deposited for patent purposes and accorded ATCC No. 67244.

As used herein, ERR means estrogen-related receptor. The acronyms, hERR1 and hERR2 refer to human estrogen-related receptors 1 and 2. These receptors are more related to steroid receptors than to the thyroid receptors, yet they do not bind any of the major classes of known steroid hormones (Giguere, *et al.*, 1988). hERR1 is encoded by plasmids pE4 and pHKA, which hav been deposited for patent purposes and accorded ATCC No. 67309 and 67310, respectively. (Neither pE4 or pHKA are

complet clones; hERR1 is constructed by joining segments from both clon s.) hERR2 is encoded by plasmid phH3 which has been deposited for patent purposes and accorded ATCC No. 40373.

As used herein, RAR means retinoic acid receptor. The acronym, hRARα, refers to human retinoic acid receptor *alpha*. hRARα is encoded by plasmid phRARα which has been deposited for patent purposes and accorded ATCC No. 40392.

As used herein, VDR means vitamin $D_3$ receptor.

As used herein, an "orphan" receptor is a protein encoded by a DNA sequence that is homologous with DNA sequences that encode known members of the steroid/thyroid superfamily of hormone receptors. The term "orhpan" denotes the fact that the ligand(s) which bind to the putative receptor are not yet known.

As used herein, element P means one of two clusters of amino acids in the steroid/thyroid receptors that can differentially control target gene specificity of the DNA binding domains. The cluster of amino acids that comprise element P for the GR subfamily (GR, MR, PR, AR) is "GSCKV"; the clusters that comprise element P for the ER subfamily ($T_3R\alpha$, $T_3\alpha$, RARα, RARβ, $VD_3R$, NGFIB, $TR_2$, *v-erbA*, ear2, ear3, *Knirps*, *Knirps-related*, ERR1, ERR2) are "EGCKA", "EGCKG", "EGCKS", and "EACKA". Also see Table 2.

As used herein, element D means one of two clusters of amino acids in the steroid/thyroid receptors that can differentially control target gene specificity of the DNA binding domains. The clusters of amino acids that comprise element D for the GR subfamily (GR, MR, PR, AR) are "AGRND" and "ASRND"; the clusters that compris elem nt P for the ER subfamily ($T_3R\alpha$, $T_3\alpha$, RARα, RARβ, $VD_3R$, NGFI-B, $TR_2$, *v-erbA*, ar2, ear3, *Knirps*, *Knirps-related*, ERR1, ERR2) are "PATNQ", "KYDSC", "KYEGK", "HRDKN", "PFNGD", "LANKD", "RGSKD", "TYDGC",

"RSNRD", "RANRN", "KNEGK", "KNNGE", "PASNE", and "PATNE". Also *see* Table 2.

As used herein, *CAT* means chloramphenicol acetyltransferase.

As used herein, luciferase means firefly luciferase. *See*, de Wet, *et al.*, (1987).

As used herein, COS means monkey kidney cells which express T antigen (Tag). *See* Gluzman, *Cell*, 23:175 (1981). As used herein, CV-1 means mouse kidney cells from the cell line referred to as "CV-1". CV-1 is the parental line of COS. Unlike COS cells, which have been transformed to express SV40 T antigen (Tag), CV-1 cells do not express T antigen. CV-1 cells are receptor-deficient cells that are also useful in the assays of the present invention.

As used herein, HRE means hormone response element. HREs are short *cis*-acting sequences (about 20 bp in size) that are required for hormonal (or ligand) activation of transcription. The attachment of these elements to an otherwise hormone-nonresponsive gene causes that gene to become hormone responsive. HREs function in a position- and orientation-independent fashion. Unlike other enhancers, the activity of the HREs is dependent upon the presence or absence of ligand. *See* Evans (1988) and the references cited therein.

As used herein, engineered HREs refer to HREs that have been recombinantly produced using genetic engineering techniques such as nucleotide substitution, deletion, *etc.* If wild-type, engineered or synthetic HREs are linked to hormone-nonresponsive promoters, these promoters become hormone responsive. *See* Evans (1988) and the references cited therein.

As used herein, synthetic HREs refer to HREs that have been synthesized *in vitro* using automated nucl otide synthesis machines. Since the HREs are only

about 20 bp in size, they are easily synthesized in this manner. If wild-type, engineered or synthetic HREs are linked to hormone-nonresponsive promoters, these promoters become hormone responsive. See Evans (1988) and the references cited therein.

As used herein, the acronym GRE means glucocorticoid response element and TRE means thyroid receptor response element. GREs are hormone response elements that confer glucocorticoid responsiveness via interaction with the GR. See Payvar, *et al.*, *Cell*, 35:381 (1983) and Schiedereit, *et al.*, *Nature*, 304:749 (1983). GREs can be used with any wild-type or chimeric receptor whose DNA-binding domain can functionally bind- (*i.e.*, activate) with the GRE. For example, since GR, MR and PR receptors can all activate GREs, a GRE can be used with any wild-type or chimeric receptor that has a GR, MR or PR-type DNA-binding domain. TREs are similar to GREs except that they confer thyroid hormone responsiveness via interaction with TR. TREs can be used with any wild-type or chimeric receptor whose DNA-binding domain can functionally bind (*i.e.*, activate) with the TRE. Both TR and RR receptors can activate TREs, so a TRE can be used with any receptor that has a TR or RR-type DNA-binding domain.

As used herein, ligand means an inducer, such as a hormone or growth substance. Inside a cell the ligand binds to a receptor protein, thereby creating a ligand/receptor complex, which in turn can bind to an appropriate hormone response element. Single ligands may have multiple receptors. For example, both the $T_3R\alpha$ and the $T_3R\beta$ bind thyroid hormone such as $T_3$.

As used herein, the word "operative", in the phrase "operativ hormon response element functionally linked to a ligand-responsive promoter and an operative reporter gene", means that the respectiv DNA sequences (represented by the t rms "hormone response element",

"ligand-responsive promoter" and "reporter gene") are operational, *i.e.*, the hormone respons elem nt can bind with the DNA-binding domain of receptor prot in (either wild-type or chimeric), the ligand-responsive promoter can control transcription of the reporter gene (upon appropriate activation by a HRE/receptor protein/ligand complex) and the reporter gene is capable of being expressed in the host cell. The phrase "functionally linked" means that when the DNA segments are joined, upon appropriate activation, the reporter gene (*e.g.*, *CAT* or luciferase) will be expressed. This expression occurs as the result of the fact that the "ligand responsive promoter" (which is downstream from the hormone response element, and "activated" when the HRE binds to an appropriate ligand/receptor protein complex, and which, in turn then "controls" transcription of the reporter gene) was "turned on" or otherwise activated as a result of the binding of a ligand/receptor protein complex to the hormone response element.

As used herein, the phrase "DNA-binding domain" of receptors refers to those portions of the receptor proteins (such as glucocorticoid receptor, thyroid receptor, mineralocorticoid receptor, estrogen-related receptor and retinoic acid receptor) that bind to HRE sites on the chromatin DNA. The boundaries for these DNA-binding domains have been identified and characterized for the steroid hormone superfamily. *See* Evans, (1988); also *see* Giguere, *et al.*, (1986); Hollenberg, *et al.*, (1987); Green and Chambon (1987); Miesfield, *et al.*, (1987); and Evans (1988).

The DNA-binding domains of the steroid hormone superfamily of receptors consist of an amino segment varying between 66 to 68 amino acids in length. This segment contains 9 cysteine residues, one of which is the first amino acid of the segment. This first Cys residu begins a motif described as Cys-$X_2$-Cys-$X_{13-15}$-Cys-

$X_2$-Cys, where X is any amino acid residue. The DNA-binding domain invariably nds with the amino acids Gly-Met.

As used herein, the phrase "ligand-binding domain region" of receptors refers to those portions of the receptor proteins that bind to ligands such as growth substances or the hormones. These boundaries of the ligand-binding domains for the steroid receptor superfamily have been identified and characterized. *See* Evans (1988).

As used herein, "mutating" means using genetic engineering techniques to alter DNA so that it is different from the "wild-type" or unmodified sequences. Useful genetic engineering techniques for altering the DNA include, but are not limited to, insertion of new nucleotides into wild-type sequences, deletion of nucleotides from wild-type sequences, and substitution of nucleotides in the wild-type sequences, for example by site directed mutagenesis.

As used herein, "mutant" DNA of the invention refers to DNA that has been genetically engineered to be different from the "wild-type" or unmodified sequences. Such genetic engineering includes insertion of new nucleotides into wild-type sequences, deletion of nucleotides from wild-type sequences, or substitution of nucleotides in the wild-type sequences, for example by site directed mutagenesis.

Use of the term "substantial sequence homology" in the present specification and claims means it is intended that DNA, RNA, or amino acid sequences which have slight and non-consequential sequence variations from the actual sequences disclosed and claimed herein ar within th scope f the appended claims. In this regard, the "slight and non-consequ ntial" sequence variations mean that the homologous sequences will be functionally equivalent to the sequences of the

invention, *i.e.*, the homologous sequences function in substantially the same manner to produce substantially the same compositions as the nucleic acid and amino acid compositions disclosed and claimed herein.

As used herein, the term "recombinantly produced" means made using genetic engineering techniques, not merely purified from nature.

The amino acids which comprise the various amino acid sequences appearing herein may be identified according to the following three-letter or one-letter abbreviations:

| Amino Acid | Three-Letter Abbreviation | One Letter Abbreviation |
|---|---|---|
| L - Alanine | Ala | A |
| L - Arginine | Arg | R |
| L - Asparagine | Asn | N |
| L - Aspartic Acid | Asp | D |
| L - Cysteine | Cys | C |
| L - Glutamine | Gln | Q |
| L - Glutamic Acid | Glu | E |
| L - Glycine | Gly | G |
| L - Histidine | His | H |
| L - Isoleucine | Ile | I |
| L - Leucine | Leu | L |
| L - Lysine | Lys | K |
| L - Methionine | Met | M |
| L - Phenylalanine | Phe | F |
| L - Proline | Pro | P |
| L - Serine | Ser | S |
| L - Threonine | Thr | T |
| L - Tryptophan | Trp | W |
| L - Tyrosine | Tyr | Y |
| L - Valine | Val | V |

Th nucl otides which comprise th various nucleotide sequences appearing herein have their

usual single-letter designations (A, G, T, C or U) used routinely in the art.

As used herein, bp means base pairs and kb means kilobase pairs.

In the present specification and claims, the Greek letters alpha (α), beta (β), gamma (γ), *etc.*, are sometimes referred to as a, b, g, *etc.*

In the present specification and claims, unless noted otherwise, temperatures are in degrees Centigrade.

DEPOSITS

Plasmids pRShGR (hGR), pRShMR (hMR), peA101 ($Ht_3\beta$) and GM*CAT*, all of which are in *E. coli* HB101, plus plasmids pE4 and pHKA (which together encode hERR1), phH3 (hERR2), pher*b*-A 8.7 (hTRα, phFA 8 (a partial clone of hTRα, and plasmid phRARα have been deposited at the American Type Culture Collection, Rockville, Maryland, U.S.A. (ATCC) under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the plasmids are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of said Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted.

The ATCC Deposit Numbers and Deposit Dates for the deposits ar as follows:

| | | |
|---|---|---|
| pRShGR (hGR) | 67200 | Sept. 9, 1986 |
| pRShMR (hMR) | 67201 | Sept. 9, 1986 |
| pE4 (hERR1*) | 67309 | Jan. 30, 1987 |

| | | |
|---|---|---|
| phHKA (hERR1*) | 67310 | Jan. 30, 1987 |
| phH3 (hERR2) | 40373 | Sept. 29, 1987 |
| GMCAT (reporter) | 67282 | Dec. 18, 1986 |
| pherb-A 8.7 (hTRα) | 40374 | Sept. 29, 1987 |
| peA101 (hTRβ) | 67244 | Oct. 22, 1986 |
| phRARα (hRARα) | 40392 | Nov. 20, 1987 |

(* means a partial clone)

(pE4 & phHKA together encode complete hERR1)

## DESCRIPTION OF THE INVENTION

In one aspect, the present invention comprises steroid/thyroid hormone receptor DNA binding domain compositions that determine target gene specificity. The compositions of the invention are selected from the group consisting of GSCKV, EGCKA, EGCKG, EGCKS, EACKA, AGRND, ASRND, PATNQ, KYDSC, KYEGK, HRDKN, PFNGD, LANKD, RGSKD, TYDGC, RSNRD, RANRN, KNEGK, KNNGE, PASNE, and PATNE.

In another aspect, the present invention is a cluster of amino acids comprised of EGxxGxxR where x is selected from the group consisting of A, R, N, D, C, Q, E, H, I, L, K, M, F, P, S, T, W, Y, and V.

In still another aspect, the present invention is a cluster of amino acids comprised of KVEGK.

In still another aspect, the present invention is comprised of mutant receptors selected from the group consisting of GTG8, GTG3A, GTG2, GTG1.

In still another aspect, the invention comprises a method for constructing a receptor that can activate both GRE and ERE sequences. The method comprises introducing a point mutation into the glucocorticoid receptor DNA sequence so that the encoded glycine at the site between $C_3$ and $C_4$ is replaced with a glutamic acid. Conversely, a point mutation is introduced into the estrogen receptor DNA sequenc so the encoded glutamic acid at the site between $C_3$ and $C_4$ is r placed with a glycine.

In still another aspect, the invention comprises a substantially pure receptor protein that can activate both glucocorticoid and estrogen response element sequences.

In still another aspect, the invention comprises methods for converting the target gene specificity of one receptor into the target gene specificity of another. According to this aspect of the invention, target gene specificity of the glucocorticoid receptor is converted to that of the estrogen receptor by changing three amino acids clustered in the first zinc finger. A single Gly to Glu change in this region produces a receptor with dual sequence responsiveness. Further replacement of five amino acids in the stem of the second zinc finger transforms the specificity to that of the thyroid hormone.

Still further the invention comprises novel assays for identifying functional ligands for orphan hormone receptors. These assays are especially useful since they avoid the necessity of constructing chimeric genes and proteins in order to search for ligands that can activate an orphan receptor. In addition, the assays of the invention do not require that the DNA sequences in question be sequenced to see if they belong to the GR or ER/TR subfamilies (although this can be done). Furthermore, as is done in the preferred assay of the invention, the presence of two reporter genes in the host cell, one operatively linked to a GRE and the other to a ERE, makes it possible to assay all unknown members of the steroid/thyroid receptor superfamily with a single assay system. In addition, a cocktail of ligands can be tested at once. If any of th ligands activate either of the reporters these ligands can be retested, *e.g.*, in smaller "cocktail" groups, and then separately. This greatly increases

the efficiency of the search for ligands for the orphan receptors.

According t one assay aspect of the invention, DNA sequences are isolated that are suspected of encoding receptor proteins. These DNA sequences are transfected into a suitable receptor-deficient host cell that has been engineered to contain at least one reporter gene functionally linked to at least one operative hormone responsive element wherein the hormone response element(s) is selected from the group consisting of wild-type, engineered or synthetic glucocorticoid response element and wild-type, engineered or synthetic estrogen response element. The transfected receptor-deficient host cell (which now contains the suspected or "orphan" receptor and at least one reporter/HRE complex) is challenged with at least one candidate ligand(s) that can potentially bind with the ligand-binding domain region of the putative receptor protein encoded by the DNA sequence in question. The induction of the reporter gene is monitored by means of changes in the protein levels of the protein encoded by the reporter gene. Finally a selection is made of ligand(s) that is capable of inducing production of the protein product of the reporter gene.

In preferred assays of the invention, the transfected host cell be a CV-1 cell which will contain at least two reporter genes, each operatively linked to a different functional HRE element. In an especially preferred form, a first reporter gene will be operatively linked to wild-type, engineered or synthetic glucocorticoid response element, and a second will be operatively linked to wild-type, engineer d or synthetic estrogen response el ment. In this form, a first reporter gene will preferably be chloramphenicol

acetyltransferase (*CAT*) and the second will preferably be firefly luciferase.

In another assay aspect of the invention, an assay is provided for identifying ligand(s) that activates an orphan receptor. According to this aspect of the invention, receptor-deficient host cells are provided with at least one reporter gene functionally linked to a preselected hormone response element. In addition, at least the DNA encoding the P region of the DNA binding domain of the orphan receptor is mutated, preferably by means of site directed mutagenesis, so that the mutated orphan receptor can activate the preselected hormone response element in receptor-deficient host cells. The host cells containing the preselected hormone response element functionally linked to at least one reporter gene are contacted with mutated orphan receptor, and then challenged with candidate ligand(s) which can potentially bind with the ligand-binding domain region of the mutated orphan receptor. Finally, induction of the reporter gene(s) is monitored as an indication of those ligand(s) which can activate the orphan receptor.

The compositions, methods and assays of the invention, plus preferred methods for making and using them, are described more fully in the Examples that follow.

## EXAMPLES

### EXAMPLE 1

### Finger Module

Previous studies on hormone receptors have shown that amino acids throughout the entire DNA-binding domain may be important for DNA-binding (Hollenberg, *t al.*, 1987). This example discloses which amino acids contribute to sequence rec gnition and are thus r sponsible for determining target g n specificity. Experimentally, it is important to know

what changes in the DNA binding domain are necessary, at a minimum, to allow the glucocorticoid receptor to recognize thyroid hormone and estrogen response elements. Between the human glucocorticoid receptor (hGR) and the human thyroid receptor *beta* (hTR$\beta$) less than half of the amino acids in this region are conserved (Fig. 1a). If the entire hGR DNA binding domain is replaced by that from the hTR$\beta$, specificity is completely switched such that hybrid GTG activates only through the TRE and no longer recognizes the GRE (Thompson, *et al.*, 1989).

In the present experiment mutant GR expression plasmids were introduced into monkey kidney CV-1 cells together with at least one reporter plasmid (*e.g.*, one of the firefly luciferase reporter plasmids) to test its target gene specificity and transactivation function. For glucocorticoid response the luciferase coding sequence was linked to the responsive MTV-LTR (MTV-LUC plasmid) (Hollenberg, *et al.*, 1988). For $T_3$-responsiveness the GREs were deleted from MTV and replaced with an oligonucleotide encoding a palindromic TRE to generate reporter $\Delta$MTV-TRE$_p$-LUC (Umesono, *et al.*, 1988; Giguere, *et al.*, 1989). Upon addition of a synthetic glucocorticoid dexamethasone, parental receptors hGRnx and GTG elicited around 2,500 and 200 fold induction over the background level from MTV-LUC and $\Delta$MTV-TRE$_p$-LUC, respectively (Fig. 2). These inductions are clearly mediated by the cognate response elements.

## EXAMPLE 2

### Receptor Identity: TRE Recognition

To identify amino acids that allow the receptors to discriminate between their cognate HREs, a vari ty of chimeric DNA binding domains were constructed. In the mutants presented in Figure 2, portions of the loops and linkers were exchanged

between the hGR and hTRβ or human retinoic acid receptor *alpha* (hRARα). These switches of Loop 1 (GTG7 and GTG32), most parts of the Linker (GTG38, GRGS, GTG36B, and GTG29), and Loop 2 and adjacent downstream portions (GTG6, GTG33, and GTG28). All of these switch mutants are active (88% to 7% of parental response) upon induction of a luciferase activity.

The interchangeable nature of the loops is remarkable in terms of dramatic changes in charge distribution with no change in target gene specificity. Furthermore, both small and large substitutions in the Linker regions are quite forgiving with no impact on promoter specificity. Reducing or expanding the spacing between the fingers from 15 (GRnx and GTG29), 17 (GTG), 19 (GRsst) and 21 (GRsstG) is again well tolerated. From phenotypes of these mutants, it is apparent that differences in the primary structure or size found in these regions are mutually permissive even between two of the most distantly related members of the receptor superfamily. Furthermore, since none of the Loop or Linker mutants described here has changed specificity, it is concluded that these amino acids apparently are not critical in discriminating between GREs and TREs or $TRE_p$ from GREs, but rather are involved in common aspects of HRE recognition and transactivation.

The modular or interchangeable nature of large segments of the finger regions does not belie the fundamental fact that there must be sequences that determine specificity. By elimination from this series of mutants, nine amino acids in two clusters were not changed and may therefore impart specificity. The first proximal (P) region follows $C_3$ and includes the three amin acid cluster GS--V in the GR and EG--G in the TR. These are part of a 10 amino acid segment that is highly cons rved and includes 6 invariant amino

acids. When these three amino acids are switched (see GTG3A in Fig. 2a and GTG36A in Fig. 2b), the resultant chimeras are nonfunctional on either MTV-LUC or ΔMTV-$PRE_p$-LUC. The second or distal (D) region of potential importance in specificity is a cluster of five amino acids between $C_5$ and $C_6$; AGRND in hGR and KYEGK in hTRβ.

Indeed, elimination of these amino acids from GTG21 to produce GTG15 yields a TRE inactive phenotype (Fig.3). Based on these observations the critical double switch and mutant GTG8 (Fig. 3) was constructed which carries an almost complete hGR DNA-binding domain with only 8 hTRβ-specific amino acids. Remarkably, this chimera induces a full conversion of target gene specificity (Figs 3 and 4H). The induction of luciferase activity on the $TRE_p$ reporter by GTG8 is as efficient as GTG itself.

Thus two noncontiguous "elements" (P and D) have been localized in the hTRβ DNA-binding domain that are sufficient to impart TRE-specific recognition in the context of an otherwise normal glucocorticoid receptor. Both elements are necessary while neither alone is sufficient. Furthermore, acquisition of the TRE specificity in this change includes a complete loss of GRE responsiveness.

## EXAMPLE 3

### Receptor Identity: ERE Recognition

Although all HREs contain a recognizable conserved motif, the TREp and the palindromic vitellogenin ERE (EREp) differ only in the spacing of what are otherwise identical half-sites (5'-TGACC-3', Fig. 1c). Furthermore, recent studies indicate that the endogenous $T_3R$ (Glass, *et al.*, 1988) as well as the *in vitro* translated product of cloned α and β TR cDNA (Thompson *t al.*, 1989) can bind with high affinity to both EREp as well as TREp. Despite this relationship, th possibility that the $T_3R$ might mediate an ERE-

dep ndent transactivation has not been carefully evaluated. To address this issue an oligonucleotide encoding the ERE was inserted into the basal ΔMTV promoter (Hollenberg, *et al.*, (1988)) to make reporter plasmids ΔMTV-ERE-LUC and ΔMTV-ERE-*CAT*.

This promoter is indeed estradiol ($E_2$) infusible because in CV-1 cells an efficient stimulation of *CAT* activity is dependent on both functional ER and the ligand $E_2$ (Fig. 4d). The transfected HER fails to activate through the GRE (Umesono and Evans, unpublished data) but does sustain a weak activator of the TREp reporter. However, in the reciprocal experiment the GTG chimera promotes activity of the ERE reporter (Figs 3a and 4d). In contrast, hGR does not induce *CAT* or luciferase activity from a EREp or TREp receptor (Figs 3a and 4b). Thus, the DNA binding domain of $hT_3R\beta$ is able to recognize both the TREp and ERE as response elements in this assay system.

Because of this close relationship between the hER and hTR mutant receptors were tested for combined ERE and TRE responsiveness to evaluate functional roles for the two specificity regions. Taking the activity of GTG as 100%, all of the TRE+ mutants in Figures 2 and 3 (GTxbaG, GTsstG, GTG32, GTG36B, GTG29, GTG28, GTG21, and GTG8) showed 70% to 650% activity upon the ERE reporter (Fig. 3). Furthermore, mutants carrying $hTR\beta$ element P but lacking element D (GTG16 and GTG3A) also showed significant inductions upon ERE even though they were inactive on the $TRE_p$ (Figs 3 and 4). These results demonstrate that $hT_3R\beta$ element P, not D, determines a positive phenotype upon the common TGACC-type half sites found in ERE and $TRE_p$. Consistently all the mutants containing GR element P (HGRNX, GTG7, GTG35, GRG8, GTG6, GTG36A, GTG5) are not responsiv to the ERE reporter (data not shown).

The phenotype of GTG3A (Figs 3c and 4g) has clearly revealed that replacement of three amino acids, forming element P, is sufficient to convert the identity of hGR DNA binding domain ($GRE^+$, $ERE^-$, $TRE^-$) into that of ER ($GRE^-$, $ERE^+$, $TRE^-$). It is noteworthy that this is a complete conversion; that is, this mutant is negative upon MTV and only positive upon ERE. The ERE responsiveness of mutant GTG3A is not restricted to the modified MTV promoter. For example, ERE-TK-LUC reporter is also regulated by GTG3A while the parental TK-LUC that lacks an oligonucleotide encoding ERE is not induced (unpublished observations).

## EXAMPLE 4

### Receptor Identity: Dual Specificity

The data presented herein have shown that element P, located in the base of Finger 1 and part of the adjacent Linker specifies the GRE-ERE responsiveness. This element includes three amino acid differences between hGR (GSCKV) and $hT_3R\beta$. Interestingly $hT_3R\beta$ and hER (EGCKA), both of which are active upon ERE, share almost identical structures when it comes to this element (Fig. 1a), indicating that these amino acid sequences are important for the recognition of ERE.

One trend of these results is that by imparting a new specificity upon a DNA-binding domain, the previous binding specificity is simultaneously lost. To determine whether individual amino acids play different roles with regard to acquisition or suppression of HRE specificity, the activity of single paired and triple mutants was examined. Although the triple mutant (GTG3A) selectively recognizes the ERE, the double mutant (GTG2) is active upon both MTV and ERE r port rs (Fig. 3c and 4f). This induction is dependent on the HRE since this receptor fails to activate eith r ΔMTV or TREp promoter (Fig. 4). An

examination of the phenotype of this mutant indicates that the last glycine in the $T_3R\beta$ P element seems to be involved in a suppression of the $GRE^+$ without a substantial impact on ERE recognition.

Based on this result the question was asked whether a single amino acid substitution would be sufficient to impart a recognizable change on receptor phenotype. Remarkably, by substituting the glucocorticoid receptor glycine or an estrogen receptor glutamic acid between $C_3$ and $C_4$ (GTG1) a receptor with dual specificity is produced. This single amino acid change leaves GRE recognition normal but fosters clear recognition of the ERE. As with GTG2 mutant, these inductions are dependent on either GREs or EREs (Fig. 3c and 4e). However, because all of these mutants lack the $T_3R\beta$ D element, they are completely inactive on the TREp.

## EXAMPLE 5

## The P and D Elements

As those skilled in the art will appreciate, the results presented in the previous Examples provide a number of unexpected conclusions concerning the mechanisms by which the nuclear receptors identify their appropriate response elements. First, the putative loops of the two zinc fingers can be exchanged between receptors without altering specificity. Second, two distinct regions, P and D, outside the loops, are critical for sequence recognition. Third, different amino acids may be involved in acquisition and loss of HRE responsiveness. Because of this, it has been possible via a single amino acid change to create a receptor with dual GRE and ERE recognition.

It is important to understand that thes results do not imply that the amino acids in the loops are not critical for sequence specific binding; rather our results show that the amino acids in the loops are

not critical in identifying the differences between response elements. Thus, it may b presumed that the commonalty of loop function indicates that they mediate recognition of common aspects of HRE structure. In contrast, the P and D elements would be involved in recognizing the variant nucleotides. This is consistent with the observation that HREs are composed of a common core sequence that can change in only a restricted number of nucleotide positions. This would suggest a putative topographical alignment between the HRE and the zinc finger such that the variable region of the HRE would always align with the P element of the finger. It can therefore be presumed that amino acids in this element make specific contacts with at least one of the variable positions in the pentomeric HRE.

In addition to primary sequence recognition there is a need to discriminate half-site spacing (ERE *vs.* TRE; Fig. 1c). As summarized in Table 1, our results indicate that the first element (P) specifies the primary nucleotide sequence of the half sites, while the second element (D) is important for the determination of the half-site spacing. In other words the second element in the hGR and hER restricts recognition to HREs with three gap nucleotides.

In respect to the amino acid sequence of the first element, all members of this receptor superfamily can be classified into either GR or ER subfamily (Table 2). The GR subfamily includes four members (GR, MR, PR, and AR (Evans, 1988); Lubahn, *et al.*, 1988); Ham, *et al.*, 1988), and all of them are able to recognize GREs, although physiological effects of each hormone are quite different (Ham, *et al.*, 1988). The other members constitute the ER/TR subfamily (ER, $T_3R\alpha$, $T_3R\beta$, $RAR\alpha$, $RAR\beta$, $VD_3R$, NGFI-B (Milbrandt, 1988), TR-2 (Chang, *et al.*, 1988), *v-erbA*, ear2 (Miyajima, *et al.*, 1988), ear3 (Miyajima, t *al.*, 1988), *knirps* (Nauber, *et al.*,

1988), *knirps-related* (Oro, *et al.*, 1988b), ERR1 and ERR2 (for reference, *see* Evans, 1988) with a slight variation. The members of this subfamily may recognize ERE, TREp, or a palindromic pair of TGACC sequences separated by five or more gap nucleotides because of the critical glutamic acid residue following $C_3$. In support of this model, our group at the Salk Institute for Biological Studies has shown that retinoic acid receptor can bind to and activate transcription through TREp.

Compared to the structural conservation of the first element, the second element is quite divergent. According to our hypothesis the second element need not bind directly to DNA but rather may determine spatial configuration of the half-sites via protein-protein interaction. One possibility is that it may represent a dimerization interface. This assumption is consistent with recent reports that the receptor DNA binding domain by itself seems to contain a dimerization signal (Kumar, *et al.*, 1988; Tsai, *et al.*, 1988).

TABLE 1

Phenotypes of the Wild-Type and Mutant DNA Binding Domains

| element P | element D | GRE | ERE | TREp |
|---|---|---|---|---|
| GR | GR | + | - | - |
| $T_3R\beta$ | $T_3R\beta$ | - | + | + |
| GR | $T_3R\beta$ | (+) | - | - |
| $T_3R\beta$ | GR | - | + | - |
| ER | ER | - | + | - |
| GR | ER | + | - | |
| ER | GR | - | + | |

The phenotypes of GR-ER hybrids are from Green, *et al.*, (1988). (+) indicates that a positive phenotype is dependent on the construct.

TABLE 2

Structure of Element P and Element D
in GR and ER/TR Subfamilies

| Receptor | Element P | Element D |
|---|---|---|
| a) GR Subfamily | | |
| GR, MR, PR, AR | GSCKV | AGRND |
| | GSCKV | ASRND |
| b) ER Subfamily | | |
| ER | EGCKA | PATNQ |
| $T_3R\alpha$ | EGCKG | KYDSC |
| $T_3R\beta$ | EGCKG | KYEGK |
| RAR$\alpha$, RAR$\beta$ | EGCKG | HRDKN |
| $VD_3R$ | EGCKG | PFNGD |
| NGFI-B | EGCKG | LANKD |
| TR2 | EGCKG | RGSKD |
| v-*erbA* | EGCKS | TYDGC |
| ear2 | EGCKS | RSNRD |
| ear3 | EGCKS | RANRN |
| *knirps* | EGCKS | KNEGK |
| *knirps*-related | EGCKS | KNNGE |
| ERR1 | EACKA | PASNE |
| ERR2 | EACKA | PATNE |

DETAILED DESCRIPTION OF THE DRAWINGS

FIGURE 1. a) Comparison of amino acid sequences among the DNA binding domains of hGR, $hT_3R\beta$, and hER (for reference, *see* Evans, 1988). Upper numbers (1 to 9) correspond to the invariant nine cysteines found in the receptor DNA binding domains. Number adjacent to the sequences indicate amino-acid positions of each receptor. Colons show the identity of amino acids between hGR and $hT_3R\beta$ or $hT_3R\beta$ and hER. The $hT_3R\beta$ DNA binding domain contains two extra amino acids in the middle of the domain. b) Predicted zinc fingers based on those for rat GR18. Numbers 1 to 9 for cysteines are as in Fig 1a. Conserved amino acids in hGR, $hT_3R\beta$, and hER are shown by one letter amino-acid code. Dots represent variable amino acids among these receptors. Two zinc ions (Zn) are chelated in a tetrahedral coordination by two clusters of four cysteines ($C_1$ to $C_4$ and $C_5$ to $C_8$), forming two "zinc fingers" (Finger 1 and Finger 2). The two fingers are separated by 15 (hGR and hER) or 17 ($hT_3R\beta$) amino acids consisting of Linker between the fingers. c) Structures of optimized hormone response elements for GR (GRE), ER (ERE), and $T_3R$ (TRE) (*See* Green and Chambon, 1988; Klock *et al.*, 1987; and Glass *et al.*, 1988). Each arrow indicates a "half site" of the HREs. These HREs consist of a palindromic pair of the half sites as shown by the arrows. Both GRE and ERE contain three gap nucleotides (nnn) between the half sites, but no such nucleotide is found in TREp (---). Dots in ERE and TREp indicate different nucleotides from those of GRE. Accordingly, ERE and TREp contain the same half site sequence.

FIGURE 2. Transactivation of luciferase reporter plasmids by mutant receptors. a) The amino acid sequence of a parental hGRnx DNA binding domain

is presented. The hGRnx contains the wild-type hGR DNA binding domain flanked by *Not*I and *Xho*I sites in the cDNA. Numbers 1 to 9 are as in Fig. 1a. Substituted or inserted amino acids in mutants GRxba and GRsst are shown. b) Structures of chimeric GR DNA binding domains carrying local $hT_3R\beta$ (GTG23, GTG7, GTG3A, and GTG6) or hRARα (GRG8) sequences. Changed amino acids are presented. Mutants are designated after the composition of their DNA binding domains; GTG23 stands for a mutant GR which has a DNA binding domain composed of both hGR and $hT_3R\beta$ sequences, and contains 23 different amino acids from the wild-type hGR sequence. c) The amino acid sequence of the $hT_3R\beta$ DNA binding domain in a hybrid receptor GTG is shown: The GTG is a hybrid GR whose DNA binding domain has been replaced by that of $hT_3R\beta$. As in a) changed amino acids are presented for GTxbaG and GTsstG. d) Chimeric $hT_3R\beta$ DNA binding domains carrying hGR sequences indicated by one letter amino-acid code. Each receptor expression plasmid was cotransfected into CV-1 cells with either glucocorticoid (MTV-LUC) or thyroid hormone (ΔMTV-TREp-LUC) responsive reporter plasmids, and the cells were cultured in the absence or presence of 100nM dexamethasone for 36 hours. Transactivation function of these receptors was judged by the induced luciferase activity from the reporter plasmids quantified as percentage of HGRNX and GTG activities upon MTV-LUC (MTV) (2,500 fold) and ΔMTV-TREp-LUC (TREp) (200 fold), respectively. The activity below the background induction level (6 fold for MTV and two fold for TREp) is indicated as -. For the activity of GTG18 on TREp, th "(2)" indicat s that the induction is not dep ndent on TREp.

Figure 2 Methods. hGRnx10 and GTG14 were constructed using known and previously published

methods. Mutant cDNAs encoding DNA binding domains of GRxba, GTG7, GTG3B, GRG8, GTG6, and GTG3A were obtain d through an oligonucleotide-directed mutagenesis (Kunkel, *et al.*, 1985) of the *Not*I-*Xho*I DNA fragment encoding the hGRnx DNA binding domain. Similarly, those for GTxbaG, GTG32, GTG36B, GTG29, GTG33, and GTG36A were made by exchanging the *Not*I-*Xho*I fragment for the $hT_3R\beta$ DNA binding domain. Mutants GRsst and GTsstG cDNAs contain a *Sst*I linker (8-mer, New England Biolabs) at filled-in *Xba*II sites of GRxba and GTxbaG, respectively. GTG23 and GTG18 were obtained by exchanging small *Not*I-*Xba*I fragments between GRxba and GTxbaG. Each of the *Not*I-*Xho*I cDNA fragments encoding mutant DNA binding domains was ligated with a large *Not*I-*Xho*I fragment obtained from pRShGRnx10. To make GTG28, *Cla*I site was introduced into $hT_3R\beta$nx14 DNA binding domain cDNA (corresponding to amino acids "ID" in the loop of the Finger 2. Because the hGRnx cDNA has the *Cla*I site at this position, the *Not*I-*Cla*I fragment of the hGRnx cDNA was replaced by that of $hT_3R\beta$nx cDNA. All mutations were confirmed by the nucleotide sequencing. Construction of reporter plasmids MTV-LUC7 and ΔMTV-TREp-LUC21 was done using previously published methods. Receptor expression plasmid (1 μg), reporter plasmid (5 μg), β-galactosidase reference plasmid (5 μg), and carrier plasmid pGEM4 (9 μg) were cotransfected. Transfection of CV-1 cells (Umesono, *et al.*, 1988) and an assay for the luciferase activity were done using published methods (de Wet, *et al.*, 1987) except that the reference plasmid pRSVβGAL was replaced by pRAS-βGAL which contains a human c-Ha-ras promoter (Ishii, *et al.*, 1985) linked to the coding sequence of β-galactosidase in a pUC-derivativ plasmid (pUCGALpA, kindly provided by Dr. Richard J.Rickles), and phenol red in the culture

media was omitted after introduction of the plasmids into the cells.

FIGURE 3. Identification of two distinct elements specifying the TREp+ and ERE+ phenotypes. Two parental DNA binding domains are presented by bold letters. In GTG identical amino acids to those in hGR are indicated by dots. Similarly, only different amino acids in mutant DNA binding domains from those of hGR are shown. The two regions (element P and D) are marked by boxes. Nomenclature of the mutants and numbers are the same as in Fig. 2. Mutant receptors were tested for transactivation function in the presence of one of the luciferase reporters carrying GREs (MTV-LUC), TREp (ΔMTV-TREp-LUC), or ERE (ΔMTV-ERE-LUC) after transient expression in CV-1 cells (± 100nM dexamethasone for 36 hours). Weak stimulation (2) by GTG15 on TREp is due to higher background activity by this mutant, and the 2% induction is not dependent on TREp. 100% induction of the luciferase activity by GTG on ΔMTV-ERE-LUC (ERE) is 10 fold, and - indicates no induction. Relative activities by TRE+ mutants shown in Fig. 2 on ERE reporter are as follows: GTG32 (90%), GTG36B (140%), GTG29 (70%), GTG33 (380%), GTG28 (650%).

Figure 3 Methods. An estrogen responsive reporter plasmid ΔMTV-ERE-LUC was constructed as follows: an oligonucleotide encoding a palindromic ERE (5'-TCAGGTCACAGTGACCTGA-3') (*see* Glass, *et al.*, 1988) was inserted into the unique *Hin*dIII site of the ΔMTV-*CAT* plasmid, in which major GREs between position -190 and -88 of MTV-LTR were deleted; this g nerated ΔMTV-ERE-*CAT*; the *CAT* gen was subsequently r plac d with the luciferase gene obtained from pSVO-A/L-A Δ5' (de Wet, *et al.*, 1987), giving ΔMTV-ERE-LUC. Mutant GTG21 is made from GTG32 by introducing

the *Cla*I site as in GTG28 (Fig. 2d). A small *Not*I-*Cla*I fragment of hGRnx was exchanged by that of GTG32-*Cla*I. GTG15 and GTG8 were obtained through site-directed mutagenesis (Kunkel, *et al.*, 1985) of the cDNAs encoding GTG21 and GTG3A DNA binding domains, respectively. Similarly GTG2 and GTG1 were made from hGRnx. Transfection and an assay of the luciferase activity were as in Fig. 2.

FIGURE 4. Induction of *CAT* activities by mutant receptors from the basal ΔMTV-*CAT* (ΔM), T3-responsive ΔMTV-TREp-*CAT* (TREp), and estrogen-responsive ΔMTV-ERE-*CAT* (ERE) reporters. Together with one of the reporters, indicated receptor expression plasmids from a) to h) were transfected into CV-1 cells. The receptors were activated by adding 100nM of dexamethasone (D) or 17$\beta$-estradiol (E2) for 36 hours. The "-" symbol indicates that the solvent ethanol was added. Structures of hGRnx, GTG, GTG8, GTG3A, GTG2, and GTG1 were presented in Figs. 2 and 3. The notation "no receptor" indicates that an expression plasmid encoding $hT_3R\beta$ in the reverse orientation was cotransfected. hER; human estrogen receptor. From e) to h), changed amino acids are shown in a schematic figure for hGR zinc fingers. The calculated % conversions in the *CAT* assays are **a)** ΔM (-) 0.4, (D) 0.5, TREp (-) 0.4, (D) 0.6, ERE (-) 0.6, (D) 1.0, **b)** ΔM (-) 0.4, (D) 0.7, TREp (-) 0.3, (D) 0.8, ERE (-) 0.4, (D) 1.2, **c)** ΔM (-) 1.8, ($E_2$) 1.6, TREp (-) 1.6, ($E_2$) 7.0, ERE (-) 1.8, ($E_2$) 78, **d)** ΔM (-) 1.4, (D), 4.6, TREp (-) 1.1, (D) 74, ERE (-) 1.9, (D) 72, e) ΔM (-) 0.5, (D) 1.3, TREp (-) 0.6, (D) 1.4, ERE (-) 1.2, (D) 20, **f)** ΔM (-) 0.8, (D) 1.8, TREp (-) 0.9, (D) 3.5, ERE (-) 1.3, (D) 96, **g)** ΔM (-) 0.5, (D) 0.7, TREp (-) 0.5, (D) 0.6, ERE (-) 1.0, (D) 88, **h)** ΔM (-) 0.7, (D) 4.3, TREp (-) 0.7, (D) 87, ERE (-) 1.0, (D) 95.

Figure 4 Methods. Reporter plasmids ΔMTV-*CAT* (Hollenberg, *et al.*, 1988), ΔMTV-TREp-*CAT* (TRE-p1M*CAT* (Umesono, *et al.*, 1988; Thompson and Evans, 1989), ΔMTV-ERE-*CAT* (see legend for Fig. 3), and an expression plasmid for hER35 were previously described. CV-1 cell transfection has been carried out as described in the legend for Fig. 2 except that the luciferase reporters were replaced by the *CAT* reporters. For the *CAT* assay (Gorman, *et al.*, 1982), cell extracts corresponding to 30 units of β-galactosidase activity (Herbomel, *et al.*, 1984) were incubated for 3 hours. Calculation of % conversion for *CAT* activity was carried out as previously described (Thompson and Evans, 1989).

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## SPECIFICATION SUMMARY

From the foregoing description, one of ordinary skill in the art can understand that the present invention discloses steroid/thyroid hormone receptor DNA binding domain compositions that determine target gene specificity. The invention further discloses methods converting the target gene specificity of one receptor into the target gene specificity of another. Still further the invention discloses novel assays for identifying functional ligands for putative or "orphan" hormone receptors. These assays are especially useful since they avoid the necessity of constructing chimeric genes and proteins in order to search for ligands that can activate an orphan receptor.

Without departing from the spirit and scope of this invention, one of ordinary skill can make various changes and modifications to the invention to adapt it to various usages and conditions. As such, these changes and modifications are properly, equitable, and intended to be, within the full range of equivalence of the following claims.

WHAT IS CLAIMED IS:

1. Element P amino acids selected from the group consisting of GSCKV, EGCKA, EGCKG, EGCKS, and EACKA.

2. Element D amino acids selected from the group consisting of AGRND, ASRND, PATNQ, KYDSC, KYEGK, HRDKN, PFNGD, LANKD, RGSKD, TYDGC, RSNRD, RANRN, KNEGK, KNNGE, PASNE, PATNE, AGRND, ASRND, PATNQ, KYDSC, KYEGK, HRDKN, PFNGD, LANKD, RGSKD, TYDGC, RSNRD, RANRN, KNEGK, KNNGE, PASNE, and PATNE.

3. Mutant receptors selected from the group consisting of GTG8, GTG3A, GTG2, GTG1.

4. A method for constructing a receptor that can activate both GRE and ERE sequences, said method comprising introducing a point mutation into: (A) the glucocorticoid receptor DNA sequence so that the encoded glycine at the site between $C_3$ and $C_4$ is replaced with a glutamic acid, or (B) the estrogen receptor DNA sequence so that the encoded glutamic acid at the site between $C_3$ and $C_4$ is replaced with a glycine.

5. A substantially pure receptor protein that can activate both glucocorticoid and estrogen response elements.

6. A substantially pure receptor protein according to Claim 5 wherein said protein is produced by the method of Claim 4.

7. A method for identifying functional ligand(s) for an orphan receptor protein, said method comprising: (A) isolating DNA sequences suspected of encoding receptor protein; (B) transfecting into a suitable receptor-deficient host cell: (1) the DNA sequences from step A, and (2) at least one reporter gen functionally linked to at least one operative hormone response element wherein said hormone response lement(s) is s l cted from the group

consisting of wild-type, engineered or synthetic glucocorticoid response element and wild-type, engineered or synthetic estrogen response element; (C) challenging the transfected host cell from step B with candidate ligand(s) which can potentially bind with the ligand-binding domain region of the orphan receptor protein encoded by the DNA sequence of step A; and (D) monitoring induction of the reporter gene(s).

8. A method for identifying functional ligand(s) for an orphan receptor protein, said method comprising: (A) isolating DNA sequences suspected of encoding receptor proteins; (B) transfecting into a suitable receptor-deficient host cell: (1) the DNA sequences from step A, (2) at least one reporter gene functionally linked to at least one operative hormone responsive element wherein the hormone response element(s) is selected from the group consisting of wild-type, engineered or synthetic glucocorticoid response element, and (3) at least one reporter gene functionally linked to at least one operative hormone response element wherein the hormone response element(s) is selected from the group consisting of wild-type, engineered or synthetic estrogen response element; (C) challenging the transfected host cell from step B with candidate ligand(s) which can potentially bind with the ligand-binding domain region of the orphan receptor protein encoded by the DNA sequence of step A; and (D) monitoring induction of the reporter gene(s).

9. A method for identifying functional ligand(s) for an orphan receptor protein (ORP), said method comprising: (A) identifying at least one functional hormone response element that is activated by th orphan r ceptor protein (ORP); (B) contacting said orphan receptor protein (ORP) in receptor-

deficient host cells with at least one reporter gen operatively linked to said functional hormone response element identified in step A; chall nging said host cells from step B with candidate ligand(s) which can potentially bind with the ligand-binding domain region of said orphan receptor protein (ORP); and (D) monitoring induction of the reporter gene(s).

10. A method according to Claim 9 A wherein said functional hormone response element is identified by (A) determining the amino acid sequence of at least the hormone response element determining portion of the DNA binding domain of said orphan receptor protein, and (B) identifying a hormone response element that is activated by the amino acids determined in step A.

11. A method for identifying functional ligand(s) for an orphan receptor protein in a cell wherein said cell contains (A) a DNA sequence suspected of encoding an orphan receptor protein, (B) at least one reporter nucleic acid sequence functionally linked to an operative hormone response element wherein the hormone response element is selected from the group consisting of wild-type, engineered or synthetic glucocorticoid response element, and (C) at least one reporter nucleic acid sequence functionally linked to an operative hormone response element wherein the hormone response element is selected from the group consisting of wild-type, engineered or synthetic estrogen response element; said method comprising challenging the receptor-deficient cell with at least one candidate ligand and monitoring induction of the reporter nucleic acid sequence(s).

12. A method of any of Claims 7-11 wherein said cell is a CV-1 cell.

13. A method according to any of Claims 7-11 wherein the reporter gene is sel cted from the group consisting of a chloramph nicol acetyltransferase (*CAT*) gene and a firefly luciferase gene.

14. A method for identifying a ligand(s) that activates an orphan receptor comprising: (A) providing in receptor-deficient host cells at least one reporter gene functionally linked to a preselected hormone response element; (B) mutating at least the DNA encoding the P region of the DNA binding domain of the orphan receptor so that the mutated orphan receptor thus produced can activate the preselected hormone response element from step A; (C) contacting receptor-deficient cells from step A with mutated orphan receptor from step B; (D) challenging said cells from step C with candidate ligand(s) which can potentially bind with the ligand-binding domain region of said mutated orphan receptor; and (E) monitoring induction of the reporter gene(s).

15. A method according to Claim 14 B wherein said P region is mutated by sight directed mutagenesis.

AMENDED CLAIMS
[received by the International Bureau on 17 September 1990 (17.09.90) original claims 7 and 8 amended; other claims unchanged (2 pages)]

1. Element P amino acids selected from the group consisting of GSCKV, EGCKA, EGCKG, EGCKS, and EACKA.

2. Element D amino acids selected from the group consisting of AGRND, ASRND, PATNQ, KYDSC, KYEGK, HRDKN, PFNGD, LANKD, RGSKD, TYDGC, RSNRD, RANRN, KNEGK, KNNGE, PASNE, PATNE, AGRND, ASRND, PATNQ, KYDSC, KYEGK, HRDKN, PFNGD, LANKD, RGSKD, TYDGC, RSNRD, RANRN, KNEGK, KNNGE, PASNE, and PATNE.

3. Mutant receptors selected from the group consisting of GTG8, GTG3A, GTG2, GTG1.

4. A method for constructing a receptor that can activate both GRE and ERE sequences, said method comprising introducing a point mutation into: (A) the glucocorticoid receptor DNA sequence so that the encoded glycine at the site between $C_3$ and $C_4$ is replaced with a glutamic acid, or (B) the estrogen receptor DNA sequence so that the encoded glutamic acid at the site between $C_3$ and $C_4$ is replaced with a glycine.

5. A substantially pure receptor protein that can activate both glucocorticoid and estrogen response elements.

6. A substantially pure receptor protein according to Claim 5 wherein said protein is produced by the method of Claim 4.

7. A method for identifying functional ligand(s) for an orphan receptor protein, said method comprising: (A) isolating DNA sequences suspected of encoding receptor protein; (B) introducing into a suitable receptor-deficient host cell: (1) the DNA sequences from step A, and (2) at least one reporter gene functionally linked to at least one operative hormone response element wherein said hormone response element(s) is selected from the group

consisting of wild-type, engineered or synthetic glucocorticoid response element and wild-type, engineered or synthetic estrogen response element; (C) challenging the transfected host cell from step B with candidate ligand(s) which can potentially bind with the ligand-binding domain region of the orphan receptor protein encoded by the DNA sequence of step A; and (D) monitoring induction of the reporter gene(s).

8. A method for identifying functional ligand(s) for an orphan receptor protein, said method comprising: (A) isolating DNA sequences suspected of encoding receptor proteins; (B) introducing into a suitable receptor-deficient host cell: (1) the DNA sequences from step A, (2) at least one reporter gene functionally linked to at least one operative hormone responsive element wherein the hormone response element(s) is selected from the group consisting of wild-type, engineered or synthetic glucocorticoid response element, and (3) at least one reporter gene functionally linked to at least one operative hormone response element wherein the hormone response element(s) is selected from the group consisting of wild-type, engineered or synthetic estrogen response element; (C) challenging the transfected host cell from step B with candidate ligand(s) which can potentially bind with the ligand-binding domain region of the orphan receptor protein encoded by the DNA sequence of step A; and (D) monitoring induction of the reporter gene(s).

9. A method for identifying functional ligand(s) for an orphan receptor protein (ORP), said method comprising: (A) identifying at least one functional hormone response element that is activated by th orphan receptor protein (ORP); (B) contacting said orphan receptor protein (ORP) in receptor-

# FIG. 1A

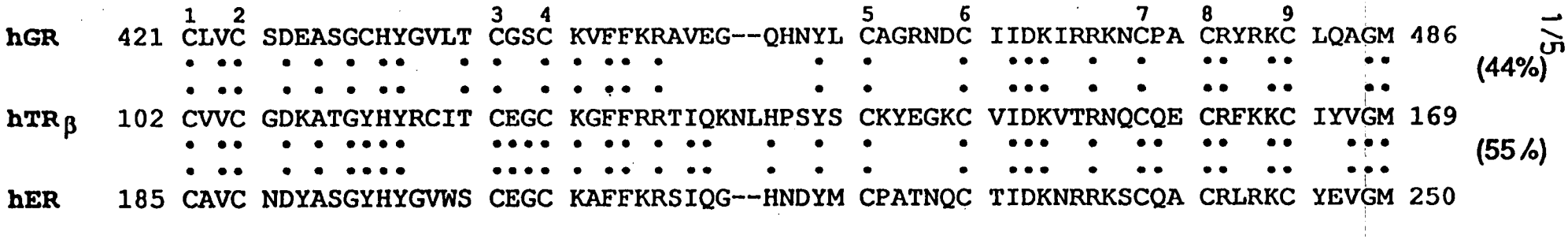

1 2 3 4 5 6 7 8 9
hGR 421 CLVC SDEASGCHYGVLT CGSC KVFFKRAVEG--QHNYL CAGRNDC IIDKIRRKNCPA CRYRKC LQAGM 486
(44%)
hTRβ 102 CVVC GDKATGYHYRCIT CEGC KGFFRRTIQKNLHPSYS CKYEGKC VIDKVTRNQCQE CRFKKC IYVGM 169
(55%)
hER 185 CAVC NDYASGYHYGVWS CEGC KAFFKRSIQG--HNDYM CPATNQC TIDKNRRKSCQA CRLRKC YEVGM 250

FIG. 1B

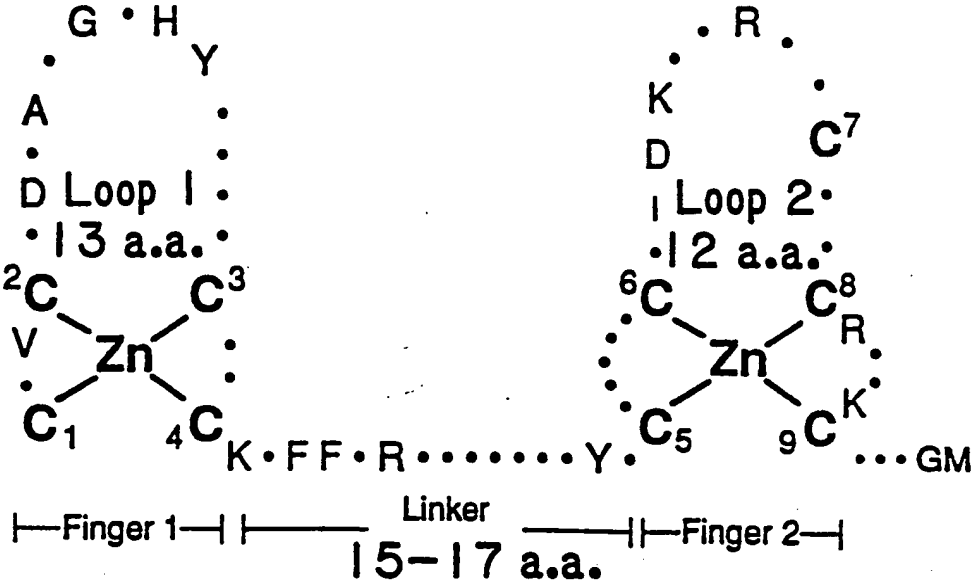


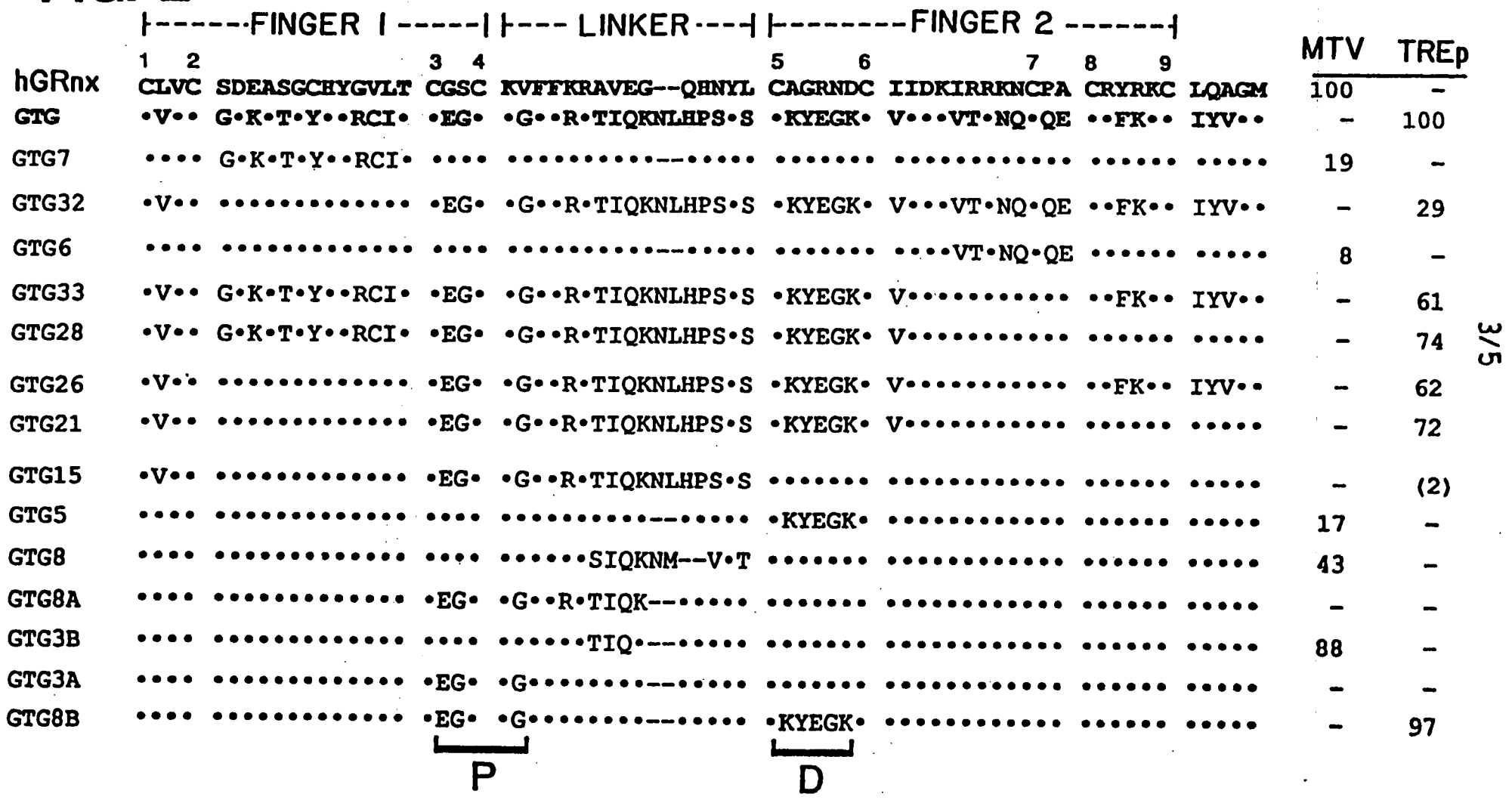

FIG. 1C

GRE : AGAACAnnnTGTTCT

ERE : AGGTCAnnnTGACCT

TREp : AGGTCA---TGACCT

# FIG. 2


FINGER 1
LINKER
FINGER 2
1 2 3 4 5 6 7 8 9
hGRnx
CLVC SDEASGCHYGVLT CGSC KVFFKRAVEG--QHNYL CAGRNDC IIDKIRRKNCPA CRYRKC LQAGM
MTV
TREp
GTG •V•• G•K•T•Y••RCI• •EG• •G••R•TIQKNLHPS•S •KYEGK• V•••VT•NQ•QE ••FK•• IYV•• 100 -
GTG7 •••• G•K•T•Y••RCI• •••• ••••••••••--••••• ••••••• •••••••••••• •••••• ••••• - 100
GTG32 •V•• •••••••••••• •EG• •G••R•TIQKNLHPS•S •KYEGK• V•••VT•NQ•QE ••FK•• IYV•• 19 -
GTG6 •••• •••••••••••• •••• ••••••••••--••••• ••••••• ••••VT•NQ•QE •••••• ••••• - 29
GTG33 •V•• G•K•T•Y••RCI• •EG• •G••R•TIQKNLHPS•S •KYEGK• V•••••••••• ••FK•• IYV•• 8 -
GTG28 •V•• G•K•T•Y••RCI• •EG• •G••R•TIQKNLHPS•S •KYEGK• V•••••••••• •••••• ••••• - 61
GTG26 •V•• •••••••••••• •EG• •G••R•TIQKNLHPS•S •KYEGK• V•••••••••• ••FK•• IYV•• - 74
GTG21 •V•• •••••••••••• •EG• •G••R•TIQKNLHPS•S •KYEGK• V•••••••••• •••••• ••••• - 62
GTG15 •V•• •••••••••••• •EG• •G••R•TIQKNLHPS•S ••••••• •••••••••••• •••••• ••••• - 72
GTG5 •••• •••••••••••• •••• ••••••••••--••••• •KYEGK• •••••••••••• •••••• ••••• - (2)
GTG8 •••• •••••••••••• •••• ••••••SIQKNM--V•T ••••••• •••••••••••• •••••• ••••• 17 -
GTG8A •••• •••••••••••• •EG• •G••R•TIQK--•••••• ••••••• •••••••••••• •••••• ••••• 43 -
GTG3B •••• •••••••••••• •••• ••••••TIQ•--•••••• ••••••• •••••••••••• •••••• ••••• - -
GTG3A •••• •••••••••••• •EG• •G••••••••--••••• ••••••• •••••••••••• •••••• ••••• 88 -
GTG8B •••• •••••••••••• •EG• •G••••••••--••••• •KYEGK• •••••••••••• •••••• ••••• - -
P
D

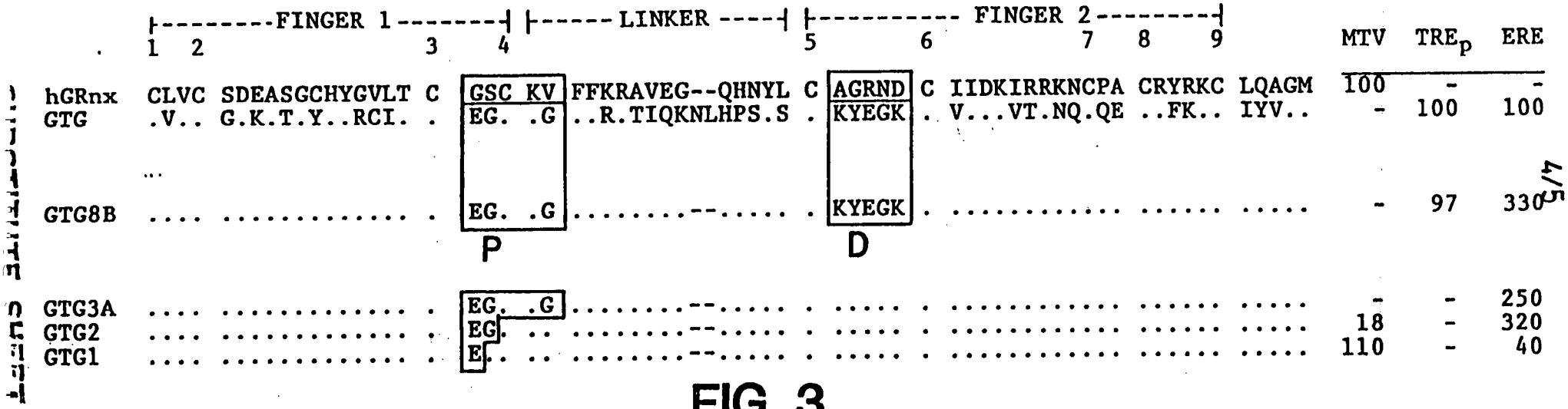

FINGER 1
LINKER
FINGER 2
1 2 3 4 5 6 7 8 9
MTV TREp ERE
hGRnx CLVC SDEASGCHYGVLT C GSC KV FFKRAVEG--QHNYL C AGRND C IIDKIRRKNCPA CRYRKC LQAGM 100 - -
GTG .V.. G.K.T.Y..RCI. . EG. .G ..R.TIQKNLHPS.S . KYEGK . V...VT.NQ.QE ..FK.. IYV.. - 100 100
GTG8B .... ..... . EG. .G ..... . KYEGK . ..... ..... ..... - 97 330
P
D
GTG3A .... ..... . EG. .G ..... . ..... . ..... ..... ..... - - 250
GTG2 .... ..... . EG. .. ..... . ..... . ..... ..... ..... 18 - 320
GTG1 .... ..... . E.. .. ..... . ..... . ..... ..... ..... 110 - 40

FIG. 3

FIG. 4A
no receptor




FIG. 4B
hGR nx




FIG. 4C
hER




FIG. 4D
GTG




FIG. 4E
GTG8




FIG. 4F
GTG3A




FIG. 4G
GTG2




FIG. 4H
GTG1

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01428

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

INT CL: C07C 229/00; 51/245; C07K 13/00; G01N 337566; 33/48
US CL: 562/433; 530/350; 436/501, 86, 87, 63

## II. FIELDS SEARCHED

Minimum Documentation Searched 7

| Classification System | Classification Symbols |
|---|---|
| US | 530/350, 399<br>562/433, 553<br>436/501, 86, 87, 63 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Intelligenetics Polypeptide Analysis System Search for Sequences of Figure I.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

| Category * | Citation of Document, 11 with indication, where appropriate, of the relevant passages 12 | Relevant to Claim No. 13 |
|---|---|---|
| X/Y | Cell, vol. 55, issued 02 December 1988, "Multiple and Cooperative Trans-Activation Domains of the Human Glucocorticoid Receptor", (Hollenberg et al, page 8) 99-906. | 1-4 |
| X/Y | Cell, vol. 55, issued 23 December 1988 "Transcriptional inhibition by a Glucocorticoid Receptor-B-Galactosidase Fusion Protein", (Oro et al), pages 1109-1114 | 1-4 |
| X/Y | Cell, Vol. 51, issued 24 December 1987, "Functional Domains of the Human Estrogen Receptor", (Kumor et al) pages 941-951. | 4-15 |
| X | Cell, vol. 46, Issued 29 August 1986, "Functional Domains of the Human Glucocorticoid Receptor", (Giguere et al) pages 645-652. | 5-15 |
| X | Nature, vol. 330, issued 17 December 1987, "Identification of a Receptor of the Morphogen Retinoic Acid", (Giguere et al), pages 624-629. | 5-15 |
| Y | Cell, vol. 55, issued 07 October 1988, "The Estrogen Receptor Binds Tightly to Its Responsive Elements as a Ligand-Induced Homodimer | 5-15 |

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 24 MAY 1990 | 20 JUL 1990 |

| International Searching Authority | Signature of Authorized Officer |
|---|---|
| ISA/US | STEPHANIE W. ZITOMER, PhD. |

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

FURTHER INF RMATION CONTINUED FROM THE SECOND SHEET

| | | |
|---|---|---|
| Y | Cell, vol. 49, issued 10 April 1987, "Colocalization of DNA-Binding and Transcriptional Activation Functions in the Human Glucocorticoid Receptor", (Hollenberg et al) pages 39-46. | 5-15 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers 1, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [4]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.